AD NUMBER

AD868151

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies and their contractors; Administrative/Operational Use; APR 1970. Other requests shall be referred to Department of the Army, Fort Detrick, Attn: Technical Release Branch/TID, Frederick, MD 21701.

AUTHORITY

BDRL ltr, 29 Sep 1971

AD

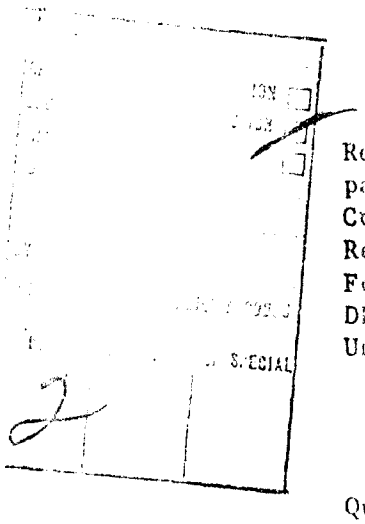
TECHNICAL MANUSCRIPT 598

CONCENTRATION OF RIFT VALLEY FEVER AND CHIKUNGUNYA VIRUSES BY PRECIPITATION WITH AMMONIUM SULFATE AND POTASSIUM ALUMINUM SULFATE

Frederick Klein
Bill G. Mahlandt
Ralph R. Cockey
Ralph E. Lincoln

APRIL 1970

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland



DDC AVAILABILITY NOTICES

Qualified requesters may obtain copies of this publication from DDC.

Foreign announcement and dissemination of this publication by DDC is not authorized.

Release or announcement to the public is not authorized.

DISPOSITION INSTRUCTIONS

Destroy this publication when it is no longer needed. Do not return it to the originator.

The findings in this publication are not to be construed as an official Department of the Army position, unless so designated by other authorized documents.

BLANK PAGE

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland 21701

TECHNICAL MANUSCRIPT 598

CONCENTRATION OF RIFT VALLEY FEVER AND CHIKUNGUNYA VIRUSES BY PRECIPITATION WITH AMMONIUM SULFATE AND POTASSIUM ALUMINUM SULFATE

Frederick Klein

Bill G. Mahlandt

Ralph R. Cockey

Ralph E. Lincoln

Process Development Division
AGENT DEVELOPMENT & ENGINEERING LABORATORIES

Project 1B563603DE32

April 1970

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

ACKNOWLEDGMENT

We acknowledge the fine cooperation and technical assistance of Howard M. Hodge.

ABSTRACT

A simple and efficient method for concentrating Rift Valley fever and chikungunya viruses is described. Ammonium sulfate and potassium aluminum sulfate are used as precipitating agents, and the precipitate is resuspended to volumes suitable for further processing and purification. Concentration of both viruses by one or the other sulfate may be obtained this way. The method permits concentration of live Rift Valley fever and chikungunya viruses about 100-fold with negligible losses of virus. Rift Valley fever virus retained a higher level of infectivity with potassium aluminum sulfate, but chikungunya virus retained a higher infectivity level with ammonium sulfate. The data indicate that serum plays an important role in the concentration of both viruses.

I. INTRODUCTION*

Except for density gradient techniques that employ ultra-high-speed centrifugation, the literature contains little on the concentration and purification of the two arboviruses Rift Valley fever (RVF) and chikungunya (Chik). It is true that certain other viruses have been concentrated by adsorption to salts and molecular gels (e.g., polioviruses on aluminum hydroxide gels,[1,2] fowl plague, influenza, Newcastle disease, and mumps viruses on calcium phosphate[3,4] or aluminum phosphate,[5,6] and poliovirus and enteroviruses by precipitation with cations[7-9]), but these concentration methods require complex equipment.

This report describes a simple procedure for concentrating RVF and Chik viruses by precipitation or adsorption to ammonium sulfate or potassium aluminum sulfate.

II. MATERIALS AND METHODS

A. VIRAL STRAIN

The small-plaque variant of the pantropic van Wyk strain of RVF virus[10] was used in this study. The origin and maintenance of this strain were described earlier.[11] The African Chik-2 strain of Chik virus was prepared from mouse brains harvested 42 hours postinfection. A 10% suspension was made in medium 199 containing 400 units of penicillin and 400 µg of streptomycin per ml.

B. VIRAL ASSAY PROCEDURES

Diluent for RVF virus was composed of one part of medium 199 and two parts of Hanks balanced salt solution (v/v) supplemented with 10% calf serum. Heart infusion broth was used for Chik virus. Fort Detrick Swiss-Webster strain mice weighing 8 to 10 g were used for titrating RVF virus assay; suckling mice less than 30 hours old were used for the Chik virus assay. Eight mice were used per dilution, and each mouse was challenged intracerebrally with 0.03 ml of viral material. Deaths were recorded for 6 days postinoculation; only those occurring after 24 hours were used in the calculation of mouse intracerebral lethal dose$_{50}$ (MICLD$_{50}$). The probit method[12] of calculating MICLD$_{50}$ values was used.

---

* This report should not be used as a literature citation in material to be published in the open literature. Readers interested in referencing the information contained herein should contact the senior author to ascertain when and where it may appear in citable form.

C. TISSUE STRAINS AND CULTURAL PROCEDURE

Two variants of Earle's L cells were used to propagate the RVF virus: (i) a selected clone designated clone 1-1 made from the L-MA line initially obtained from Dr. Donald Merchant, University of Michigan, Ann Arbor, Michigan, and (ii) the L-DR line obtained from Dr. William F. Daniels, Fort Detrick, Frederick, Maryland. Growth medium for L-MA clone 1-1 was 199 medium supplemented with 0.5% Bacto-Peptone. Medium for the L-DR cells was composed of Eagle's minimum essential medium (EMEM) supplemented with 10% bovine serum as modified by Daniels et al.[13] These cultures were PPLO-free after plating on Mycoplasma agar medium.* Cell cultures were grown routinely in antibiotic-free medium. Suspension cultures of cells were grown in the shake flask system.[14]

Prior to infection with RVF virus, tissue cells near the peak of the log phase were diluted to approximately 2 x 10^5 cells/ml in the same type of growth medium used to produce the culture. Multiplicities of inoculum (MOI) of either 0.01 or 0.001 were seeded directly into the tissue cell culture. Flasks were incubated at 37 C for 72 hours on a reciprocating shaker (100 3-inch strokes/min).

A variant of Earle's L cell, CCLI 929, was used to propagate Chik virus in a monolayer system. PPLO-free cells were grown in medium 199 supplemented with 5% calf serum and containing 100 units of penicillin and 100 μg of streptomycin per ml. At the time of virus inoculation, the growth medium was replaced with fresh medium, with or without serum as appropriate. An MOI of 2.5 was used to inoculate the 24-hour-old monolayer culture in Roux bottles. After 48 hours' incubation at 37 C, the fluids were pooled and titrated in suckling mice.

D. CONCENTRATION OF VIRUS

Cellular debris was removed from the RVF virus suspension by low-speed centrifugation (480 x g for 10 min) in a Sorvall RC-2 centrifuge at 4 C, and the cold (4 C) supernatant fluid was concentrated on the same day. Cell debris was not a problem with Chik virus grown in monolayer, so the centrifugation step was not necessary before concentration.

Two techniques were employed. In the first, stock solution of 100% saturated ammonium sulfate [$(NH_4)_2SO_4$] was prepared, pH was adjusted to 7.4 with 1 N sodium hydroxide, and to each original starting volume of cold (4 C) supernatant fluid, stock was added intermittently in small amounts with pH maintained at 7.4 with sodium bicarbonate. After 15 min, the cold (4 C), treated supernatant fluid was centrifuged for 30 min at 6,000 x g in the SS-34 rotor of a Sorvall RC-2 centrifuge operating at 4 C. The volume of the supernatant fluid was measured and titrated. The precipitate was resuspended in an equal volume of medium 199 plus 10% bovine serum.

* Grand Island Biological Company, Grand Island, N.Y.

The second concentration technique employed alum (potassium aluminum sulfate) [$KAl(SO_4)_2 \cdot 12H_2O$] to precipitate the virus from the supernatant fluid. A 5% stock solution of alum was prepared, and the pH was adjusted to 7.4 with 1 N sodium hydroxide. To each 100-ml volume of cold (4 C) supernatant fluid, 2 ml of 5% stock solution were added. (Final concentration of alum was 0.1%.) The alum stock solution was added intermittently while the pH was kept at 7.4 with sodium bicarbonate. After 15 min, the cold (4 C), treated supernatant fluid was centrifuged in the SS-34 rotor of a Sorvall RC-2 centrifuge operating at 4 C for 30 min at 6,000 x g. The volumes of the supernatant fluid and precipitate were measured, titrated, and retained or discarded as described previously.

E. ANALYSIS OF DATA

The techniques utilized to concentrate both RVF and Chik viruses provide certain known values: i.e., the volume and concentration of the (i) original virus suspension, (ii) supernatant fluid, and (iii) precipitate. The concentrations were calculated on both per-milliliter and total-volume bases. Results reported show the degree of concentration achieved and percentage recovery of infectivity.

III. RESULTS AND DISCUSSION

The results reported here on both Chik and RVF viruses show some direct evidence that the virions are: (i) associated with the serum protein and (ii) readily concentrated by precipitation with one or the other of the sulfates employed.

Table 1 summarizes the concentration effect of ammonium sulfate and Table 2 that of alum. Both viruses reacted similarly, in that larger infectivity losses occurred in serum-free than in serum-supplemented medium. However, greater concentration of RVF virus occurred with alum than with ammonium sulfate; the converse was true for Chik virus. In all cases, by either of the two precipitation techniques, the volume was reduced 98% or more. However, two of five attempts to precipitate Chik virus produced in serum-free medium resulted in no precipitation by ammonium sulfate (Table 1).

TABLE 2. CONCENTRATION OF RIFT VALLEY FEVER AND CHIKUNGUNYA VIRUSES BY POTASSIUM ALUMINUM SULFATE PRECIPITATION

Medium	Sample	Mean	Standard Deviation
Serum-Free	RVF (10 replications)		
	Infectivity, $\log_{10}$ $MICLD_{50}$ per ml		
	Original suspension	6.9	0.38
	Precipitate	7.2	0.56
	Concentration achieved		
	Volume, %	98	
	Recovered infectivity, % of total volume	23.0	16.8
	Chik (6 replications)		
	Infectivity, $\log_{10}$ $MICLD_{50}$ per ml		
	Original suspension	7.7	0.32
	Precipitate	8.3	0.33
	Concentration achieved		
	Volume, %	98	
	Recovered infectivity, % of total volume	12.8	2.8
Serum-Supplemented	RVF (17 replications)		
	Infectivity, $\log_{10}$ $MICLD_{50}$ per ml		
	Original suspension	6.4	0.19
	Precipitate	8.1	0.19
	Concentration achieved		
	Volume, %	99	
	Recovered infectivity, % of total volume	175.5	23.4
	Chik (12 replications)		
	Infectivity, $\log_{10}$ $MICLD_{50}$ per ml		
	Original suspension	8.1	0.16
	Precipitate	8.7	0.19
	Concentration achieved		
	Volume, %	98	
	Recovered infectivity, % of total volume	17.5	7.8

The concentration effect of ammonium sulfate precipitation shows that both viruses grown in serum-free medium (Table 1) demonstrated substantial losses in infectivity. Although the volume of the virus suspension was effectively reduced to 2% of the original volume, only 5.2 and 28.5% of the initial infectivity were recovered in the precipitates for RVF and Chik viruses, respectively. Similar results were obtained when RVF virus was propagated in serum-supplemented medium and concentrated by this process. The initial volume was reduced to 1%, but only 12.9% of the original infectivity was recovered in the precipitate. In contrast, when Chik virus was propagated in serum-supplemented medium and precipitated by this process, a high degree of concentration was achieved. The initial volume was reduced to 2%, with 73.5% of initial infectivity recovered; however, 1 to 5% of the original infectivity was found in the supernatant fluid. This demonstrated an overall loss of infectivity of approximately 21 to 25%. Table 3 shows an example of a single run in which 3,000 ml of virus suspension were reduced to 60 ml without loss of infectivity.

TABLE 3. EXAMPLE OF CONCENTRATION ACHIEVED ON LARGE VOLUMES OF RIFT VALLEY FEVER AND CHIKUNGUNYA VIRUS SUSPENSIONS

	Original Viral Suspension	Concentrated Viral Suspension
RVF[a]:		
Volume, ml	600	13[b]
Log_{10} $MICLD_{50}$/ml	6.5	8.8
Log total $MICLD_{50}$[c]	9.3	9.9
Chik[d]:		
Volume, ml	3,000	60
Log_{10} $MICLD_{50}$/ml	9.0	11.4
Log total $MICLD_{50}$	12.5	13.2

a. Precipitated by alum.
b. Volume represents the original precipitate plus an equal volume of suspending medium.
c. Volume x $MICLD_{50}$/ml = total $MICLD_{50}$.
d. Precipitated by ammonium sulfate.

The original titer of Chik viral suspensions had no effect on the efficiency of the process; starting materials with both high (9.0 $\log_{10}$ $MICLD_{50}$/ml) and low (7.7 $\log_{10}$ $MICLD_{50}$/ml) infectivity titers were concentrated as high as 100-fold without appreciable loss of infectivity. This concentration technique proved to be rapid and very convenient for concentrating Chik virus to volumes suitable for storage and preparative ultra-centrifugation.

When ammonium sulfate proved ineffective in concentrating RVF virus, it was postulated that the properties of the two viruses differed either in the isoelectric points of their protein or in the structure of the protein of the virus particles. Therefore, a different precipitant was tested.

Data on the concentration of these two viruses by alum precipitation are given in Table 2. Both viruses propagated in serum-free medium decreased substantially in volume, but, again, exhibited large losses both in infectivity and in recovery of initial infectivity in the precipitate.

In contrast, RVF virus was concentrated by alum precipitation in serum-supplemented medium (Table 2). The volume of RVF virus culture was reduced to 1% with greater than 100% recovery of initial infectivity in the precipitate. Table 3 shows an example of a single run in which 600 ml of virus suspension were reduced to 13 ml with no loss of infectivity. Infectivity titrations of the supernatant fluid showed a retention of 25 to 50% of initial infectivity and suggested more virus present than in the initial culture. Note that quantitation of results in this paper is based on infectivity and not on number of virus particles present in the culture material. Polson and Levitt[15] presented evidence suggesting that RVF virus produces infective particles that are not sedimentable, are filamentous, and contain lipoid material. This interpretation or that of incomplete virus[16] may explain our results. Conversely, Chik virus lost 82.5% of its initial infectivity after alum precipitation in contrast to 25% by ammonium sulfate precipitation.

Certain advantages of the two precipitation techniques employed here are: (i) a large volume of material can be concentrated to a volume (Table 3) suitable for storage and preparative ultra-centrifugation, and (ii) virus infectivity is not lost during the concentration process under conditions favorable for that particular virus: RVF virus by alum precipitation and Chik virus by ammonium sulfate precipitation in serum-containing medium. We cannot explain why one salt (ammonium sulfate) concentrates Chik virus and another salt (potassium aluminum sulfate) concentrates RVF virus. It is apparent, however, that serum does play the important role in the concentration of both viruses. Work is in progress to assess the degree of purification as well as the mechanisms of reaction.

LITERATURE CITED

1. Sabin, A.B. 1931. Experiment on the purification and concentration of the virus of poliomyelitis. J. Exp. Med. 56:307-317.

2. Schaeffer, M.; Brebner, W.B. 1933. Purification of poliomyelitis virus. Arch. Pathol. 15:221-226.

3. Elford, W.J.; Chu, D.M.; Dawson, I.M.; Dudgeon, V.A.; Fulton, F.; Smiles, J. 1948. Physical properties of the viruses of Newcastle disease, fowl plague, and mumps. Brit. J. Exp. Pathol. 29:590-595.

4. Salk, J.E. 1941. Partial purification of the virus of epidemic influenza by adsorption on calcium phosphate. Proc. Soc. Exp. Biol. Med. 46:709-712.

5. Frimmhagen, L.H.; Knight, C.A. 1959. Column purification of influenza virus. Virology 8:198-208.

6. Miller, H.D.; Schlesinger, R.W. 1955. Differentiation and purification of influenza viruses by adsorption on aluminum phosphate. J. Immunol. 75:155-160.

7. Hampil, B.; Melnick, J.L.; Wallis, C.; Brown, R.W.; Braye, E.T.; Adams, R.R., Jr. 1965. Preparation of antiserum to enteroviruses in large animals. J. Immunol. 95:895-908.

8. Grossowicz, N.; Mercado, A.; Goldblum, N. 1960. A simple method for concentration of live and formaldehyde-inactivated poliomyelitis virus. Proc. Soc. Exp. Biol. Med. 103:872-874.

9. Midulla, M.; Wallis, C.; Melnick, J.L. 1965. Enterovirus immunizing antigens in the form of cation-stabilized and concentrated virus preparations. J. Immunol. 95:9-12.

10. Boyle, J.J. 1967. Some characteristics of plaque variants of Rift Valley fever virus. Amer. J. Vet. Res. 125:1027-1031.

11. Walker, J.S.; Carter, R.C.; Klein, F.; Snowden, S.E.; Lincoln, R.E. 1969. Evaluation of factors related to growth of Rift Valley fever virus in suspended cell cultures. Appl. Microbiol. 17:658-664.

12. Finney, D.J. 1952. Statistical methods in biological assay. Hafner Publishing Co., New York.

13. Daniels, W.F.; Parker, D.A.; Johnson, R.W.; Schneider, L.E. 1965. Controlled pH and oxidation-reduction potential with a new glass tissue culture fermentor. Biotechnol. Bioeng. 7:529-533.

14. Weirether, F.J.; Walker, J.S.; Lincoln, R.E. 1968. A precise method for replicating suspension cultures of mammalian cells. Appl. Microbiol. 16:841-844.

15. Polson, A.; Levitt, J. 1963. A slowly sedimenting infectious component of Rift Valley fever virus. J. Hyg. 61:451-469.

16. Mims, C.A. 1956. Rift Valley fever virus in mice: IV. Incomplete virus; its production and properties. Brit. J. Exp. Pathol. 37:129-143.

Unclassified
Security Classification

DOCUMENT CONTROL DATA - R & D
(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

Department of the Army
Fort Detrick, Frederick, Maryland, 21701

2a. REPORT SECURITY CLASSIFICATION: Unclassified
2b. GROUP

3. REPORT TITLE

CONCENTRATION OF RIFT VALLEY FEVER AND CHIKUNGUNYA VIRUSES BY PRECIPITATION WITH AMMONIUM SULFATE AND POTASSIUM ALUMINUM SULFATE

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)

5. AUTHOR(S) (First name, middle initial, last name)

Frederick (NMI) Klein Ralph R. Cockey
Bill G. Mahlandt Ralph E. Lincoln

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
April 1970	15	16

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
b. PROJECT NO. 1B563603DE32	Technical Manuscript 598
c. Task-Work Unit 04-003	9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
d. DD 1498 Agency Access. DA OL 0305	CMs 6667 AMXFD-AE-T 49813

10. DISTRIBUTION STATEMENT

Qualified requesters may obtain copies of this publication from DDC. Foreign announcement and dissemination of this publication by DDC is not authorized.
Release or announcement to the public is not authorized.

11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
Process Development Division	Department of the Army Fort Detrick, Frederick, Md., 21701

13. ABSTRACT

A simple and efficient method for concentrating Rift Valley fever and chikungunya viruses is described. Ammonium sulfate and potassium aluminum sulfate are used as precipitating agents, and the precipitate is resuspended to volumes suitable for further processing and purification. Concentration of both viruses by one or the other sulfate may be obtained this way. The method permits concentration of live Rift Valley fever and chikungunya viruses about 100-fold with negligible losses of virus. Rift Valley fever virus retained a higher level of infectivity with potassium aluminum sulfate, but chikungunya virus retained a higher infectivity level with ammonium sulfate. The data indicate that serum plays an important role in the concentration of both viruses.

14. Key Words

Rift Valley fever virus
Chikungunya virus
Concentration
Precipitation
Potassium aluminum sulfate
Ammonium sulfate

DD FORM 1 NOV 65 1473 REPLACES DD FORM 1473, 1 JAN 64, WHICH IS OBSOLETE FOR ARMY USE.

Unclassified
Security Classification